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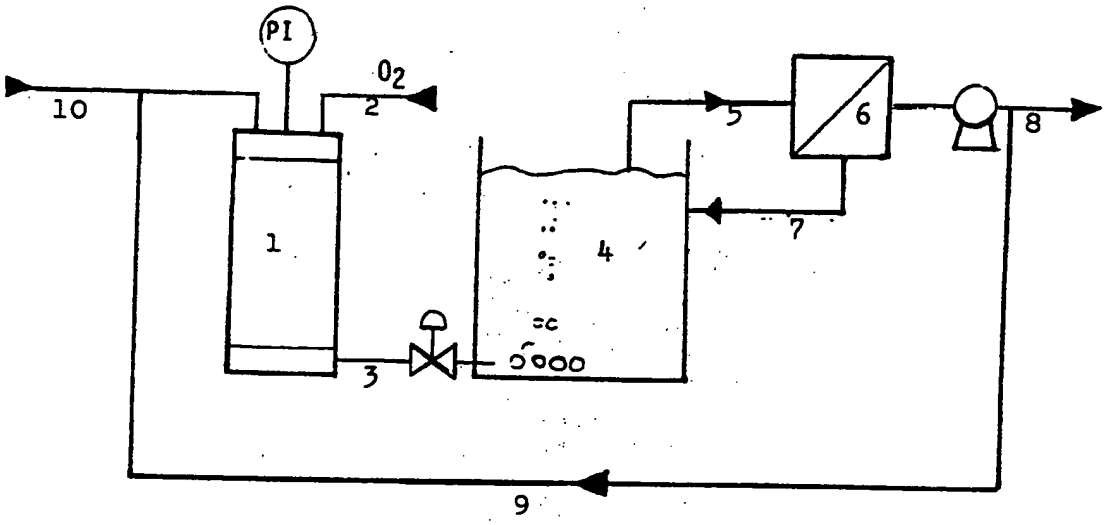

(57) Abstract

The present invention relates to a method and apparatus for supplying cultured cells with gaseous nutrients, whereby said nutrients are delivered in the form of microbubbles, with a size chosen such that most of the bubbles are dissolved and consumed before reaching the surface of the culture medium. Preferably the microbubbles are produced using a saturator.

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Method for culturing cells

The present invention relates to a method and apparatus for supplying cultured cells with gaseous nutrients, wherein the gaseous nutrients are delivered in the form of bubbles to a reactor comprising culture medium and cells in or on micro-carriers or hollow fibers, or in suspension.

Many ways are known to grow cells in culture. Until recently most of these methods related to micro-organisms such as yeasts and bacteria which are quite tolerant regarding their culture-conditions.

Recently, methods for growing more sensitive cell types such as mammalian cells, plant cells and certain micro-organisms have been developed.

Large scale culture of cells has proven to be a suitable method for the production of pharmaceutically important biochemicals, as well as for producing certain cells which are endproducts themselves.

Important biochemicals include, among others, monoclonal antibodies, recombinant proteins, cytokines etc.

Vaccins, artificial pancreas and artificial livers may be examples of cells useful as endproducts.

From a cell-culture point of view there are two kinds of cells: cells which are able to grow in suspension and cells which are anchorage dependent.

Cells which are anchorage dependent will not proliferate unless attached to a surface, a requirement "suspension cells" do not have.

For anchorage dependent mammalian cells many culture methods have been described. These include, among others, anchoring the cells in a multiplate propagator, on a special film, in hollow fibers or on microcarriers.

For mammalian and other sensitive cells, usually the methods described for micro organisms are used in large scale cell cultures. But, unlike yeasts and bacteria, mammalian cells and plant cells are much more sensitive to mechanical forces such as shear, which occur upon stirring of the culture medium and upon supplying the culture medium with gaseous nutrients. Also, though anchorage dependent cells themselves may be cultured more or less protected from shear forces, their products, which may be sensitive to shear as well, are not. Moreover, microcarriers or anchorage dependent cells growing on microcarriers are often also sensitive to shear. Therefore the large scale cell culture of mammalian or plant cells, as well as the large scale culture of cells producing shear-sensitive biochemicals or culture methods using microcarriers, have so far achieved limited success at best.

This invention relates to the supply of gaseous nutrients to culture media comprising cells or cells on microcarriers in suspension. Usual methods of supplying gaseous nutrients to such media are culturing in aerated stirred tankreactors and culturing in reactors comprising columns of bubbles of gaseous nutrients.

These two methods have two important disadvantages:

1) The already mentioned shear forces to which the suspension cells are very sensitive and which are caused by the relativily high liquid velocities which occur when using these methods.

2) A very large fraction of the volume of the gaseous nutrients escapes from the liquid surface, which leads to the formation of foam. The foam layer will entrain the cells or microcarriers, resulting in cell death, therefore anti-foam agents have to be added to the medium.

The addition of anti-foam agents may lead to decreased cell growth as well as to a decreased production of biochemicals and may hamper down-stream processing.

Moreover, pharmaceutical products are subject to stringent purity demands and therefore the anti-foam agents have to be separated from the product in an additional purification steps.

The present invention provides a method and an apparatus for supplying culture media with gaseous nutrients which do not have the aforementioned disadvantages.

The invention relates to a method of providing a bioreactor comprising a culture medium and cells (optionally on/in microcarriers) with bubbles of gaseous nutrients wherein substantially all of the bubbles are dissolved and most of the gaseous nutrients consumed before penetrating the fluid surface.

For non-stirred bioreactors it can be mathematically deduced that the initial diameter of the bubbles according to the invention has to confirm to the following formula:

$$d_{bo} < \sqrt[4]{\frac{230.\ D\ \Delta C\ H_r\ \eta}{g\ \Delta\rho\ C_{mol}}}$$

wherein D = diffusion coefficient of gaseous nutrients in the medium, [m^2/s]

ΔC = difference between the theoretical concentration of gaseous nutrient in medium saturated with bubbles and the actual concentration of gaseous nutrient in the medium [$mole/m^3$]

η = viscosity of the medium and cells in suspension [Pa.s],

$\Delta\rho$ = difference in density [kg/m^3],

C_{mol} = molar concentration [$mole/m^3$] and

H_r = height of the column of liquid in the reactor [m].

d_{bo} = initial diameter of the bubbles [m].

This mathematical deduction is given in appendix 1.

When gaseous nutrients are delivered to a culture medium according to the method of the invention, there will be no formation of foam because the bubbles do not reach the surface. Neither will there be high liquid velocities, because according to Stokes' law bubbles with a relatively small diameter rise only slowly.

For a typical bioreactor with a liquid column of about 1 meter height it can be calculated that the bubbles should have an initial diameter equal to or smaller than about 200 μm. The bubbles according to the invention are further referred to as microbubbles.

The above does not apply to bioreactors in which the medium is stirred.

Apart from rising to the surface according to Stokes law the microbubbles are subject to forces which move them in other directions. The moving liquid will entrain the microbubbles if its mean velocity is higher than the rising velocity of the microbubbles.

This is the case for all practical applicable bioreactors.

Usually, the effect of stirring is that bubbles have a longer residence time in the medium. This can be explained by several observations. First, the bubbles reaching the surface may become entrained in a downward liquid flow and therefore not break through the surface. Secondly, the bubbles which do reach the surface need some time to break through that surface. They may even need to associate with other bubbles before they lead to foam. During this time that they are "hanging" below the surface they may also become entrained in a downward liquid flow.

Since stirring generally enlarges the mean residence time of microbubbles in a certain bioreactor compared to the residence time of microbubbles is a non-stirred bioreactor, the given formula for non-stirred reactors gives an estimation for a "safe" size of microbubbles which will not lead to foam in stirred reactors with the same column height of medium.

But obviously, if the residence time of microbubbles in stirred reactors is greater than in non-stirred reactors, microbubbles may be chosen larger than calculated according to that formula.

Because there are many parameters which influence the movement and therefore the residence time of the microbubbles in the turbulent system which governs stirred bioreactors a precise calculation of the maximum size of the microbubbles cannot be made.

However, an estimation can be made of the required average bubble diameter based upon the residence time in the medium.

This relation can be mathematically deduced to be

$$d_{bo} = \frac{1,6\ k_L\ \Delta C.t_r}{C_{mol}}$$

This mathematical deduction is given in Appendix II.

A suitable method of producing these microbubbles can be arrived at by using a saturator.

A saturator is an external column separated from the cell column reactor comprising culture medium which is practically free of cells. Herein the culture medium is saturated with gaseous nutrients at a pressure greater than atmospheric pressure (for instance 5 bar). The saturated medium is transfered to the bottom of the cell culture reactor where it releases the gaseous nutrients as microbubbles, because of oversaturation at this lower pressure. The formation of microbubbles from an oversaturated liquid has been described in Perry R.H., Green D., 1988, Perry's Chemical Engineers' Handbook, 6th edition, Mc.Graw-Hill Bookco. The microbubbles were so far only produced for studying flotation behavior of these bubbles, which means they have to rise to the surface.

In a preferred embodiment the gaseous nutrient delivered to the medium as microbubbles is pure oxygen.

The above described method of oxygenation has an advantage over normal external oxygenation, because at an over pressure of for instance 5 bar, 6 times as much oxygen can be dissolved in the medium. Thus, the oxygen supply can be provided within the fresh medium supply, or, depending on the rate of consumption, in a recirculation loop, with a flow at least six times lower than with known methods.

The methods according to the invention are also very suitable for supplying cultures of anchorage dependent cells with gaseous nutrients. Especially when the anchorage dependent cells are grown attached to carriers which are kept in suspension, such as cells attached to microcarriers.

The invention also relates to an apparatus for carrying out the methods according to the invention. The apparatus usually comprises a reaction vessel, provided with a means for supplying gaseous nutrients, such as a saturator. Although other methods of providing microbubbles are also included. For continuous cell culturing, the reaction vessel will also be provided with an outlet for culture medium and cells which will lead to a separator where the medium and the cells are separated from the waste products and possibly the end product and recirculated together with new medium into the reaction vessel.

The medium may also be separated from the cells and be lead through the saturator to be supplied with gaseous nutrients. The reaction vessel may be supplied with a stirring device although this is not absolutely necessary. Of course, in continuous processes new medium may also be provided separately from the recirculated medium. An apparatus according to the invention is represented in fig. 1.

In fig. 1 a reaction vessel (4) is represented, provided with an inlet (3) for medium saturated with gaseous nutrients through a saturator (1). The reaction vessel is also provided with an outlet (5) for cells and medium from which in a first separator (6) the cells are separated and lead back to the vessel (7). In this particular embodiment a part of the medium is then recirculated (9) to the saturator and mixed with fresh medium (10) just before entering the saturator. At (8) "old" medium is withdrawn.

This particular apparatus is intended to be used for a continuous process. The person skilled in the art will be able to design an apparatus for batch-wise cell growth without departing from the spirit of the invention.

The invention will be more specifically described in example 1 and 2.

Example 1

In an apparatus (a 10 l bioreactor) as described in fig. 1 CHO cells (Chinese Hamster Ovary cells; 1.8×10^{10} cells/l) are cultured on microcarriers (Cytodex 3). The apparatus is provided with a stirring device which stirs at low shear rates (100 rpm).

The concentration of oxygen is measured with an oxygen electrode and recorded. The oxygen is provided by a saturator at a pressure of 5 bar. Regulation of the amount of oxygen delivered to the desired value is carried out by adjusting the flow through the saturator. The microcarriers were separated from the flow through the saturator by filtration with a screen filter with pores of 44 μm diameter. The culture medium comprises DMEM/F12 with 5% FCS (Fetal Calf Serum). The reactor is kept at a temperature of 37 ^{o}C.

Example 2

In a 10 liter fermentor, equipped with an oxygen saturator as described in fig. 1 and example 1, rec.CHO-cells were grown in high density macroporous microcarriers (Cultispher GD) in a continuous perfusion culture. The perfusion rate was 40 liter/day and the medium recirculation rate for oxygenation was 4 liter/hr. A steady state cell density of 3.10^{10} cells/liter was maintained during 2.5 months.
Despite the high oxygen concentration in the microbubbles and possible "shear" effects caused by cell-microbubble collision, the long term cultivation of cells in the presence of microbubbles appeared to be harmless with regard to cell growth and product formation rate.
A microcarrier settler was placed inside the fermentor to obtain a microcarrier-free medium for the recirculation flow through the saturator. The static zone in the settler was 12 cm high.
No foam formation was observed in both the mixed culture compartment (200 rpm) and the static settling compartment during cultivation at a dissolved oxygen tension of 50% air saturation. Apparently, the microbubbles are dissolved before they reach the fluid surface.
During cultivation at a dissolved oxygen tension of 80% air saturation, foam formation was observed in the settler but not in the mixed compartment.
Obviously, the microbubbles were small enough to be kept in suspension by stirring until they were completely dissolved and consumed.

The residence time in the settler was, however, under these conditions too short so that dissolution of the microbubbles was incomplete and bubble penetration through the fluid surface could be observed as foam formation.

The results show that:

1. long term oxygenation with microbubbles is effective and not detrimental to cell growth and product formation
2. a bubble diameter which is suitable in a static culture seems to be also suitable in a mixed culture under otherwise comparable conditions.

Results

The oxygen concentration could be regulated within 10% of the desired value (= 0.1 mM). Despite the presence of 5% FCS no formation of foam occurred, all microbubbles dissolved and were consumed before reaching the surface.

Appendix I

Mathematical Deduction (I)

Assumptions

1) The liquid phase mass transfer coefficient arises from Sh = 2, which is valid for a spherical bubble in a stagnant fluid. It therefore gives a safe estimation of the time required for dissolution of the bubble.
2) C_{mol} does not depend on the height
3) ΔC does not depend on the height: the liquid is ideally mixed and a pressure gradient is not taken into account
4) The bubbles are dissolved at 80% of the height of the liquid column

5) The bubbles rise as rigid spheres at a velocity calculated according to Stokes' law.

Notation

Sh = Sherwood number [-] $=k_L.d_b/D$

C_{mol} = molar gas concentration [mole/m^3]

ΔC = driving force for mass transfer, i.e. difference between the concentration of the gaseous component near the bubbles and the bulk concentration [mol/m^3]

ϕ_m = molar flow [mole/s]

k_L = mass transfer coefficient [m/s]

D = diffusion coefficient [m^2/s]

d_b = bubble diameter [m]

A = bubble area [m^2]

t = time [s]

v = velocity [m/s]

g = gravitational acceleration [m/s^2]

η = viscosity [Pa.s]

V_b = bubble volume [m^3]

H_r = height of the reactor [m]

$\Delta\rho$ = density difference between liquid and gas [kg/m^3]

The mass transfer equation for one bubble is:

$$\phi_m = k_L.A.\Delta C,$$

assume: $Sh = 2 \rightarrow k_L = \frac{2D}{d_b}$ together with $A = \pi.d_b^2$ gives :

$$\phi_m = 2\pi\ D\ d_b\ \Delta C$$

The mass balance for one bubble is:

$$\frac{d(V_b.C_{mol})}{dt} = -\phi_m$$

therefore; using $V_b = \frac{\pi}{6} d_b^3$

$$\frac{d(d_b)}{d_t} = \frac{-4D\,\Delta C}{C_{mol}\, d_b}$$

integration:

$$\int_{d_{bo}}^{d_b} d_b\, d(d_b) = \int_{o}^{t} \frac{-4\, D\, \Delta C}{C_{mol}}\, dt$$

gives

$$d_b^2 = d_{bo}^2 - \frac{8\, D\, \Delta C}{C_{mol}}\, t \qquad (1)$$

From equation (1) the total dissolution time can be derived:

$$d_b \to 0 \;\rightarrow\; t_{dis} = \frac{C_{mol}\, d_{bo}^2}{8\, D\, \Delta C} \qquad (1b)$$

According to Stokes' law the rising velocity for one bubble is:

$$v(d_b) = \frac{d_b^2\, g.\Delta\rho}{18\, \eta} \qquad (2)$$

The mean velocity is defined as:

$$\bar{v} = \frac{1}{t_{dis}} \int_0^{t_{dis}} v(t)dt \qquad (3)$$

Through combination of (2) and (1a) v(t) can be expressed as:

$$v(t) = \frac{g.\Delta\rho}{18\eta} \left(d_{bo}^2 - \frac{8\ D\, \Delta C}{C_{mol}}\, .t\right) \qquad (4)$$

The mean velocity is therefore:

$$\bar{v} = \frac{g.\Delta\rho}{18\eta} \left(d_{bo}^2 - \frac{4\ D\, \Delta C}{C_{mol}}\, .t_{dis}\right) \qquad (5)$$

Combination of (5) and (1b) gives

$$\overline{v} = \frac{g.\Delta\rho d_{bo}^2}{36\ \eta} \qquad (6)$$

Given a certain height of the liquid column (H_r) this leads to the rising time (t_r)

$$t_r = \frac{36\ H_r.\eta}{d_{bo}^2\ g.\Delta\rho} \qquad (7)$$

The bubbles have to be dissolved before reaching the surface so $t_{dis} < t_r$

Assume $t_{dis} = 0.8\ t_r$

Combining (1b) and (7) gives:

$$\frac{C_{mol}\ d_{bo}^2}{8\ \ D\ \Delta C} = 0.8x\ \frac{36\ H_r.\ \eta}{d_{bo}^2\ g\Delta\rho} \qquad (8)$$

thus

$$d_{bo} = \sqrt[4]{\frac{230.D\ \Delta C\ H_r\ \eta}{g\ \Delta\rho\ C_{mol}}} \qquad (9)$$

For H_r = 1 m,
D = 2.10^{-9} m^2/s
η = $1.06\ 10^{-3}$ $Pa.s^{-1}$
ΔC = 0.95 $mole/m^3$
C_{mol} = 44.6 $mole/m^3$
g = 10 m/s^2
$\Delta\rho$ = 1000 kg/m^3

it can be calculated that d_{bo} = 180 μm

Another way to arrive at an estimate for the initial bubble size is:

Appendix II
Mathematical Deduction (II)

The mass transfer equation for one bubble is:

$$\phi_m = k_L \, A \, \Delta C \qquad (1)$$

k_L is assumed to be constant for bubble diameters smaller than 800 μm.

The mass balance for one bubble reads:

$$\frac{d(V_b \cdot C_{mol})}{dt} = - \phi_m \qquad (2)$$

Combining eqn. 1 and 2 and insertion of

$$A = \pi d_b^2$$

$$V_b = \frac{\pi}{6} \cdot d_b^3$$

and rearrangement yields:

$$d(d_b) = - \frac{2 \, k_L \cdot \Delta C}{C_{mol}} \cdot dt \qquad (3)$$

Integration with the boundary condition $d_b = d_{bo}$ for $t = 0$ results in:

$$d_b = d_{bo} = - \frac{2 \, k_L \cdot \Delta C}{C_{mol}} \cdot t \qquad (4)$$

and the following expression for the total dissolution time ($d_b = 0$):

$$t_{dis} = \frac{d_{bo} \cdot C_{mol}}{2 k_L \cdot \Delta C} \qquad (5)$$

According to Stokes' law the rising velocity for one bubble is:

$$v(d_b) = \frac{d_b^2 \, g \cdot \Delta \rho}{18 \, \eta} \qquad (6)$$

The mean velocity is defined as:

$$\bar{v} = \frac{1}{t_{dis}} \int_0^{t_{dis}} v(t)dt \qquad (7)$$

By combination of eqn. 6 and 4, $v_{(t)}$ can be expressed as:

$$v_{(t)} = \frac{g.\Delta\rho}{18\eta} \left(d_{bo} - \frac{2\, k_L.\Delta C.t}{C_{mol}}\right)^2 \qquad (8)$$

integration and insertion of eqn. 5 yields:

$$\bar{v} = \frac{g.\Delta\rho\, d_{bo}^2}{54\eta} \qquad (9)$$

Static culture

Assuming that the bubbles have to be dissolved at 80% of the height of a static culture, the initial bubble diameter can be calculated from

$$0{,}8\ H = \bar{v}.t_{dis} \qquad (10)$$

insertion of eqn. 9 and 5 and rearrangement leads to

$$d_{bo} = \sqrt[3]{\frac{86.4 x H_r x \eta x k_L x \Delta C}{g x \Delta\rho x\, C_{mol}}} \qquad (11)$$

Example

For H = 1 m

η = 0.67 x 10^{-3} Pa.s

ΔC = 0,90 mol/m^3

k_L = 10^{-4} m/s

g = 9,8 m/s^2

$\Delta\rho$ = 1000 kg/m^3

C_{mol} = 39,4 mol/m^3

the maximal initial bubble diameter is calculated to be

d_{bo} = 240 μm

Based on the previous assumptions an estimate for the initial bubble size in a mixed (stirred) culture can be arrived at.

Mixed culture

For a mixed culture, the residence time of the bubbles must be equal to the total dissolution time:
In order to make a "safe" estimate:
Assume $0,8\ t_r < t_{dis}$

Insertion in equation 5 yields the following expression for the initial bubble diameter:

$$d_{bo} = \frac{1,6\ k_L.\Delta C.t_r}{C_{mol}} \qquad (12)$$

Example

For $k_L = 10^{-4}$ m/s
$\Delta C = 0,90$ mol/m^3
$C_{mol} = 39,4$ mol/m^3
$t_r = 80$ s
the maximal initial bubble diameter is:
$d_{bo} = 290\ \mu m$

Claims

1. Method for supplying cells in a bioreactor at least partially filled with medium with gaseous nutrients, wherein the gaseous nutrients are delivered in the form of bubbles, characterized in that the residence time of the bubbles is equal to their dissolution time in the medium.

2. Method according to claim 1, characterized in that the medium is non-stirred and that the bubbles are dissolved at a maximum of 80-95% of the height of the medium column in the bioreactor.

3. Method according to claim 2, characterized in that the initial diameter of the bubbles (d_b) conforms to

$$d_{bo} = \sqrt[4]{\frac{230.\ D\ \Delta C H_r\ \eta}{g\ \Delta\rho\ C_{mol}}}$$

wherein D = diffusion coefficient [m^2/s]
ΔC = driving force for mass transfer, i.e. difference between the concentration of the gaseous component near the bubbles and the bulk concentration [mol/m^3]
η = viscosity [Pa.s]
$\Delta\rho$ = density of the medium minus the density of the gas bubbles [kg/m^3]
C_{mol} = molar gas concentration [$mole/m^3$]
H_r = height of the column of liquid in the reactor [m].

4. Method according to claim 2 or 3, characterized in that the height of the liquid column is 1 meter and that the initial diameter of the bubbles is equal to or smaller than 250 μm.

5. Method according to claim 1, characterized in that the medium is stirred and that the initial bubble diameter conforms to

$$d_{bo} = \frac{1,6\ k_L.\Delta C.t_r}{C_{mol}}$$ wherein

k_L = mass transfer coefficient [m/s]

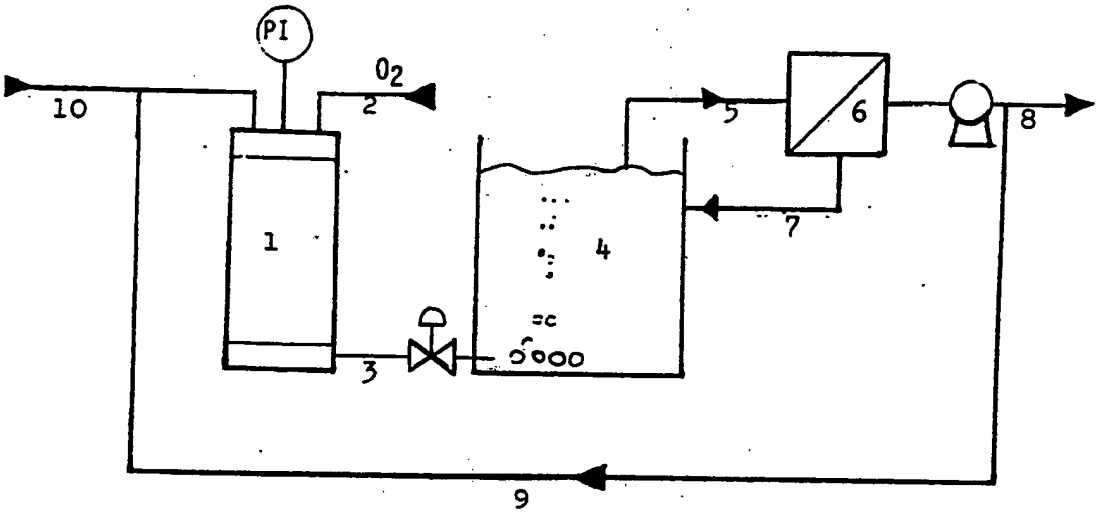
ΔC = driving force for mass transfer, i.e. difference between the concentration of the gaseous component near the bubbles and the bulk concentration [mol/m^3]

C_{mol} = molar gas concentration [mole/m^3]

t_r = residence time.

6. Method according to claim 5, characterized in that the initial diameter of the bubbles is equal to or smaller than 300 μm.

Fig. 1



INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 91/00001

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 M 3/02, C 12 M 1/04

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 M

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	US, A, 3968035 (HOWE) 6 July 1976 see claims 1,2,4; figure 1 --	1-6
X	Patent Abstracts of Japan, vol. 10, no. 219 (C-363)(2275), 31 July 1986, & JP, A, 6158582 (ADVANCE RES. & DEV. CO. LTD) 25 March 1986 see the whole abstract --	1-6
X	Patent Abstracts of Japan, vol. 12, no. 213 (C-505)(3060), 17 June 1988, & JP, A, 6314687 (MARUBISHI BAIOENJI K.K.) 21 January 1988 see the whole article --	1-6
X	US, A, 4654305 (BARNETT et al.) 31 March 1987 see claims 1-8; figure 1	1-6

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17th April 1991	21. 05. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. PEIS

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9100001 SA 43679

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 07/05/91 The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 3968035	06-07-76	None	
US-A- 4654305	31-03-87	None	